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Amer. J. Vet. Res. Vol. 52 (5) 1991. Frenkel et. al. pages 759-763 Vet. Immunol. Immunopathol. Vol. 8 (1-2) 1985. O'Donoghue et. al. pages 83-92 J. Egypt. Med. Assoc. Vol. 52 (11/12) 1969. Akkad & Mandour pages 942-948

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# (54) Sarcocystis vaccine:lyophilized bradyzoites

(57) For vaccination, lyophilized preparations of inactivated bradozooids/bradyzoites, of the sarcocystis genus are combined with a pharmaceutically acceptable carrier and preferably encapsulated for oral administration. The bradyzoites are preferably extracted from bovine heart with trypsin, separated by density gradient and detected by immunofluoresence.

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# METHOD OF PRODUCING A VACCINE AGAINST SARCOCYSTIS GENUS TOXIN AND THE PRODUCT OBTAINED THEREBY

The present invention relates to a method of producing an efficient vaccine for the prophylactic treatment and prevention of the infection caused by the toxin of the Sarcocystis protozoa in the hosts of the parasite, as well as by toxins which are closely related in chemical structure and physiological effect to such toxin.

Vaccines are biological preparations based on live, dead, or attenuated microorganisms, as well as on the products of their metabolism, which possess antigenic activity.

The microorganisms for these preparations are obtained by growing cultures in suitable medium or tissues for each specific strain, which are then attenuated or killed when harvested. Vaccines may be mixed, i.e. prepared from diverse strains. "Polyvalent" means when several vaccines are administered together. For example, "tetravalent" vaccines are effective against polio, diphtheria, whooping cough and tetanus. Vaccines may also be referred to as "toxoid", that is, they are vaccines that contain as an antigenic agent an inactivated toxin. The present invention relates to such toxoid vaccines.

The evidence of the infection with the parasite, known as sarcocystis in man, is usually an accidental finding in the course of histopathological examinations. Protozoa of the Sarcocystis genus behave like an enzoonosis, and it has been reported all over the world that they are a causal agent of many pathologies in man. This parasite mainly affects lower strata people with deficient nutrition although any person may be susceptible to it.

Protozoa of the Sarcocystis genus were reported for the first time in 1843 when a researcher by the name of Miescher found tubules in the skeleted muscle of a house mouse. Doctors Rommel and Heidorn in 1972 found that the protozoa's life cycle was heterogeneous, with the asexual phase in the prey, the intermediate host, and the sexual phase in the pillager, the final host. At present, 122 species of the protozoa have been identified and in 56 of these two hosts are known. From the zoonosis point of view, those developing the asexual phase in humans are interesting, the final hosts of which are unknown to date, and the two which develop the sexual phase in humans which are known.

Considering all scientific knowledge at the world level concerning multiple sclerosis, lateral and amyotrophic sclerosis and the syndrome of chronic fatigue, there is no report of any link between a sarcocystis-like protozoa and these diseases. According to a review of the literature in the past months, we have found that multiple sclerosis is considered to be closely linked to alterations in the immune system, mainly with alterations of the Tcd 4 lymphocytes.

Notwithstanding what has been expressed previously we have found that infection due to Sarcocystis gives rise to symptoms such as muscle spasms, intermittent diarrhoea and chronic fatigue, even to multiple sclerosis. In our research, it has been determined that the pathogeny of this organism is caused by the toxin from Sarcocystis genus. It is desirable for this toxin to be isolated so as to be inactivated but in such a way that it preserves its antigenic capacity, in order to obtain a specific immune-induced response from the part of the molecule immunologically responsive to a vaccine based on this toxin.

The present invention is based on research developed by us on infections due to toxigenic Sarcocystis, which are able to provide the toxin (Sarcocystine). It has been found that the toxin has the capacity to alter the normal function of blood microcirculation of B and T lymphocytes and the coagulation factors of blood, producing a chronic passive congestion with extravasation of liquids and solids in the form of oedema and/or haemorrhage in the different organs that are irrigated through blood microcirculation.

In the central nervous system (CNS), because the blood never comes in contact with the myelin, thanks to the system of the hematoencephalic barrier (HB), it is clear that if a patient presents with Sarcocystine, then oedemas and/or haemorrhages will be produced in the CNS. This lead us to conclude that the toxin is capable of breaking through the HB, and of increasing the blood lymphocyte count by its mitogenic capacity. In addition the parasite toxin has the capacity to increase the concentrations of the tumoral necrosing factor. When the toxin is administered in experimental infections it has been possible to reproduce demyelinating lesions, even developing Central Nervous System dysfunctions, hemiplegia, paraplegia and quadriplegia. In order to control and reverse these symptoms, treatments have been used to destroy the parasite, eliminating the source of the toxin (Sarcocystine), and permitting the detoxification of the organism or the elimination of the toxin already existing in the body. Thus, it can be concluded that the aforementioned pathological symptoms are caused by the toxic agent produced by the metabolism of a Sarcocystis-like protozoa toxin.

In accordance with the present invention there is provided a vaccine against Sarcocystine toxin comprising lyophilized

inactivated bradozooids of the Sarcocystis protozoa and a pharmaceutically acceptable carrier therefor.

The present invention also provides a method of producing a vaccine for immunisation against infections produced by Sarcocystis protozoa comprising the steps of:-

(a) inoculating Sarcocystis protozoa into a living animal,

(b) extracting the bradozooids of the protozoa from the animal,

(c) inactivating the bradozooids, and

(d) incorporating the inactivated bradozooids into a pharmaceutically acceptable carrier for administration as a vaccine.

The preferred features of the present invention are as set out in the subsidiary claims attached to this description.

In order to obtain protozoa with the optimum characteristics to produce a vaccine in accordance with the present invention the following preferred method is used:-

(a) The Sarcocystis protozoa is inoculated into an intermediate host animal, usually a young bull, in an amount such as to achieve a good count of bradozooids in the animal's muscles,

(b) The parasite bradozooids are extracted from the muscle tissue by artificial digestion by pancreatic trypsin and they are then purified by density gradients with Percol (1072-1080 mg/cc), using immunofluorescent techniques to

verify the presence of bradozooids of Sarcocystis in the extract,

(c) The extracted parasite is inactivated by chemical and/or physical methods, such as:-

- The known chemical methods using quaternary ammonium compounds.
- The known physical methods using a temperature decrease down to from -10º to 2ºC over a period of 12 hours, and

(d) Then stabilising the inactivated parasite by lyophilization.

Percol is colloidal polyvinyl pyrrolidone "PVP" coated silica particles which are known in the art for the purpose of cell separation.

It has been found, however, that the immunological reaction to the inactivated bradozooids on their own can be rather poor. Preferably isolated Sarcosystine toxin itself is added, and a method will now be described which allows the isolation of the toxin called Sarcosystine, produced by a parasite of the Sarcocystis genus which may later be used in the production of toxoids or vaccines and of specific antibodies against the said toxin. In the preferred method starting from a specific amount of heart muscle from a bovine animal with Sarcocystis cysts and using that to contaminate a canine animal, after the development of the disease in the canine animal, a percentage of its blood is extracted and, by means of a centrifugation process, the serum is separated and then purified by dialysing it against a sterile physiological saline solution, and holding it at a specific temperature, whereby the isolated toxin can then be obtained. This isolation method is

described and claimed in our co-pending application filed simultaneously herewith under reference "SJW/35849.GBA"

Specifically, the isolated toxin is preferably prepared by the following steps:-

A) Contaminating a canine animal with 250 grams of heart muscle of a bovine animal containing Sarcocystis cysts and not containing any other aetiologic agent.

B) Allowing the canine animal to develop sarcocystosis to the point where its absolute lymphocyte count reaches a minimum of $2.0 \times 10^9$ lymphocytes per litre, which usually occurs between 50 to 80 days after contamination.

C) Bleeding the animal to obtain the Sarcocystis genus protozoa usually up to 5% of its total blood volume, which blood is collected in a siliconed 500 millilitre conical glass container known as an "Erlenmeyer" flask without anticoagulant and having its interior sterile.

D) Separating the serum by centrifugation at 800 g for 20 minutes in sterile centrifuge tubes, in order to eliminate the corpuscular blood components and obtain the blood serum, regardless of any small amount of haemolysis that may occur.

E) Dialyzing the serum obtained against a sterile physiological saline solution composed of 9 grams of sodium chloride in 1000 cubic centimetres of distilled water for 36 to 48 hours, keeping the temperature between 18 and 25 degrees centigrade and shaking continuously.

At this stage we have the toxin isolated in the physiological saline solution with which the dialysing was effected and with a minimum purity of 80%. The remaining steps are:-

F) Drying the dialysed solution at a temperature of 37 degrees centigrade in order to remove the water and obtain the toxin concentrate.

G) Storing the dried toxin in sterile amber flasks and keeping them at room temperature in a cool dry place to avoid losing the strength of the dried toxin.

In order to get an optimum product for immunization a formulation is preferably made as follows:-

(i) The isolated toxin in its dried form as described above is mixed with the lyophilized parasite in its dried form as described above in a 2:1 w/w proportion to form a pre-mixture,

(ii) This pre-mixture is mixed 1:2 w/w with at least one pharmaceutically acceptable pure sugar in its solid form,

(iii) This mixture is then encapsulated so as to be in a form suitable to be administered orally.

One or two such capsules can be taken in a single dose for effecting immunization since the toxin is absorbed in the intestine, without being modified, thereby reaching the circulatory system as an antigen with a good antigenic activity, and with the zooid contents of the parasite in the vaccine inducing a local and humural response against the parasite.

The present invention provides a vaccine which is effective in humans against the Sarcocystine toxin and toxins of a like kind.

Experiments with male New Zealand rabbits have already established the viability of the present invention.

CLAIMS:

1. A vaccine for immunisation against infections produced by Sarcocystis protozoa comprising lyophilized inactivated bradozooids of the Sarcocystis protozoa and a pharmaceutically acceptable carrier therefor.

2. A vaccine as claimed in claim 1 including Sarcocystine toxin in its isolated form.

3. A vaccine as claimed in claim 2 wherein the ratio of the lyophilized inactivated bradozooids to the isolated toxin is 1:2 w/w, respectively, the isolated toxin being in its dried form.

4. A vaccine as claimed in any of the preceding claims wherein at least one pharmaceutically acceptable sugar is present.

5. A vaccine as claimed in any one of the preceding claims when in capsule form suitable for oral administration.

6. A vaccine for immunisation against Sarcocystine toxin substantially as hereinbefore described.

7. A method of producing a vaccine for immunisation against infections produced by Sarcocystis protozoa comprising the steps of:-

(a) inoculating Sarcocystis protozoa into a living animal,

(b) extracting the bradozooids of the protozoa from the animal,

(c) inactivating the bradozooids, and

(d) incorporating the inactivated bradozooids into a pharmaceutically acceptable carrier for administration as a vaccine.

8. A method as claimed in claim 7 wherein the protozoa are inoculated into a young bull.

9. A method as claimed in claim 7 or claim 8 wherein the bradozooids are extracted from the animal's muscle tissue by enzymatic digestion.

10. A method as claimed in claim 9 wherein the enzyme is pancreatic trypsin.

11. A method as claimed in any one of claims 7 to 10 wherein the extracted bradozooids are purified using known density gradient methods.

12. A method as claimed in any one of claims 7 to 11 wherein the bradozooids are extracted with the aid of known immunofluorescence techniques.

13. A method as claimed in any one of claims 7 to 12 wherein the extracted bradozooids are inactivated by subjecting them to a chemical reaction.

14. A method as claimed in claim 13 wherein the chemical reaction is with a known tertiary ammonium compound.

15. A method as claimed in any one of claims 7 to 12 wherein the extracted bradozooids are inactivated by subjecting them to a physical process.

16. A method as claimed in claim 15 wherein the physical process is cooling down to a temperature of from -10ºC to 2ºC and holding at that temperature for 12 hours.

17. A method as claimed in any one of claims 7 to 16 including the step of lyophilizing the inactivated bradozooids.

18. A method as claimed in any one of claims 7 to 17 wherein the inactivated bradozooids are mixed with isolated Sarcocystine toxin to form a pre-mix.

19. A method as claimed in claim 18 wherein the ratio of bradozooid component to isolated toxin component in the pre-mix is 1:2 w/w, respectively, the isolated toxin being in its dried form.

20. A method as claimed in claim 18 or claim 19 wherein the pre-mix is mixed with at least one pharmaceutically acceptable pure sugar in its solid form.

21. A method as claimed in claim 20 wherein the ratio of pre-mix component to sugar component is 1:2 w/w, respectively.

22. A method as claimed in any one of claims 7 to 21 including the step of encapsulating the vaccine in a form suitable for oral administration.

23. A method as claimed in claim 7 substantially as hereinbefore described.

24. A vaccine when produced by a method as claimed in any one of claims 7 to 23.

The Patent Office

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**Application No:** GB 9603303.0 **Examiner:** Dr J Houlihan
**Claims searched:** 1-24 **Date of search:** 24 April 1996

## Patents Act 1977
## Search Report under Section 17

**Databases searched:**

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:

UK Cl (Ed.O): A5B (BAA, BAC)

Int Cl (Ed.6): A61K 9/19, 39/002, 39/012

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**Documents considered to be relevant:**

| Category | Identity of document and relevant passage | Relevant to claims |
|---|---|---|
| Y | Amer. J. Vet. Res. Vol. (52) 5 1991 *"Prospective vaccine prepared from a new mutant of Toxoplasma gondii for use in cats"* Frenkel J K *et. al.* pages 759-763, especially paragraph 3 of the discussion | |
| Y | Vet. Immunol. Immunopathol. Vol. 8 (1-2) 1985 *"Atttempted immunisation of swine against acute sarcocystosis using cytozoite-derived vaccines"* O'Donoghue P J *et. al.* pages 83-92 | |
| A | J. Egypt. Med. Assoc. Vol. 52 (11/12) 1969. *"On some pharmacological and toxological effects of a protozoan toxin "sarcocystin"* " Akkad I N E & Mandour A M pages 942-948 | |

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|---|---|---|---|
| X | Document indicating lack of novelty or inventive step | A | Document indicating technological background and/or state of the art. |
| Y | Document indicating lack of inventive step if combined with one or more other documents of same category. | P | Document published on or after the declared priority date but before the filing date of this invention. |
| & | Member of the same patent family | E | Patent document published on or after, but with priority date earlier than, the filing date of this application. |

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| Y | Document indicating lack of inventive step if combined with one or more other documents of same category. | P | Document published on or after the declared priority date but before the filing date of this invention. |
| & | Member of the same patent family | E | Patent document published on or after, but with priority date earlier than, the filing date of this application. |